Analytica Chimica Acta, 283 (1993) 361–366
Elsevier Science Publishers B.V., Amsterdam

Rapid separation of fluorescein derivatives using a micromachined capillary electrophoresis system

D. Jed Harrison, Zhonghui Fan and Kurt Seiler

Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2 (Canada)

Andreas Manz and H. Michael Widmer

Forschung Analytik, Ciba-Geigy, CH-4002 Basel (Switzerland)

(Received 8th September 1992; revised manuscript received 19th January 1993)

Abstract

Using micromachining techniques electrophoresis systems consisting of sample injectors and separation capillaries have been fabricated in planar glass structures. A device with a total capillary channel length of 13.9 cm was used to inject and separate fluorescein and fluorescein sulfonate dyes within 3 min, with an injector to detector distance of 6.0 cm, and an applied field of 520 V/cm. A similar device with a capillary length of 1.6 cm effected injection and separation within 4 s for an injector to detector length of 0.75 cm at an applied field of 1875 V/cm. Injected sample volumes of 60 pl or less were readily injected and analyte detected at 10 μM concentrations.

Keywords: Electrophoresis; Sensors; Fluorescein; Capillary electrophoresis; Micromachining

The automation of chemical analysis has been achieved using a systems approach [1–5], through the coupling of sample handling with separation and detection to give an analytical instrumentation package. While robotics provides one route for this, the use of flow systems as in flow injection analysis (FIA) [6], coupled with separation and detection methods [5] to facilitate sample handling, pretreatment and analysis is another promising method. Systems prepared using this strategy have been designated total chemical analysis systems (TAS) [4]. Examples include gas chromatograph-based monitors for trace analysis in air [7], on-line glucose analyzers [3] and a supercritical fluid chromatograph coupled with a sampling system for process control [8].

Miniaturization of the TAS concept (μ-TAS) to incorporate all sample handling operations on a monolithic structure about the size of a silicon chip is an attractive concept [4,5]. Such a device could conceivably function as a dip-type probe, useful for analysis of samples in a complex matrix in a manner comparable to that of a highly selective chemical sensor [5,9]. Miniaturization also offers advantages for many separation methods, as has been well established, and integration of capillaries, detectors and injectors could increase separation efficiency by reduction of dead volumes. Practical benefits of μ-TAS devices include considerable reductions in solvent and reagent use, smaller sample volumes, and increased speed of analysis [5,9,10].

We have designed a μ-TAS system based on capillary electrophoresis (CE). It consists of an injector and a capillary column integrated together on a glass chip. It utilizes electroosmotic pumping to drive solution flow in the injector

Correspondence to: D.J. Harrison, Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2 (Canada).

manifold and electrophoresis for separation of sample components [9–12]. The structure is fabricated in glass using integrated circuit technology for micromachining of the capillary channels. We have recently shown that separations can be effected in such a planar structure [9,10], and that the efficiencies obtained are similar to those for electrophoresis in fused silica capillaries [9]. In this report we examine the efficiency of separation using a more sophisticated detector design, and establish that sample injection and separation can be achieved within only a few seconds using these devices. Quantification of the picolitre scale volumes injected has also been demonstrated.

EXPERIMENTAL

A pH 8.0 buffer was prepared from 0.050 M boric acid (reagent grade) and 0.050 M tris(hydroxymethyl)amino methane (Tris, reagent grade). Stock solutions of 100 μM fluorescein (Na^+ salt, Aldrich) and fluorescein-5 (and -6) sulfonate (Na^+ salt, Molecular Probes, Eugene, OR) were used to prepare 10 and 50 μM dye solutions. Elemental analysis of the sulfonated dye was inconclusive as to purity, because of the presence of Na^+ and the dye's hygroscopic character. Solutions were filtered with 0.2 μm membranes and sparged with He.

The capillary electrophoresis TAS (CETAS) device, Fig. 1, was fabricated by Mettler AG (Switzerland) under contract using a proprietary lithographic process [9]. The sample channel length between reservoir 2 and the intersection was 16.7 cm, while the separation channel from reservoir 3 to the intersection was 13.9 cm. About 91% of a voltage applied between reservoirs 1 and 3 was dropped between the intersection and reservoir 3. The distance from injection point (i.e., intersection) to detection point was 6.0 cm for CETAS in these experiments. Device JH-1 was fabricated at the Alberta Microelectronic Centre in fine-annealed Pyrex glass, using a photolithographic process to be described elsewhere [11]. The bottom glass plates were etched to form channels. Top plates with holes located appropriately were then bonded to these to form capillaries, using a high temperature process (620 to 650°C) described previously [9,11]. The channel length from reservoir 1 to 2 was 0.80 cm, and from reservoir 3 to 4 was 1.6 cm. The distance from injection point (i.e. intersection) to detection point was 0.75 cm for JH-1.

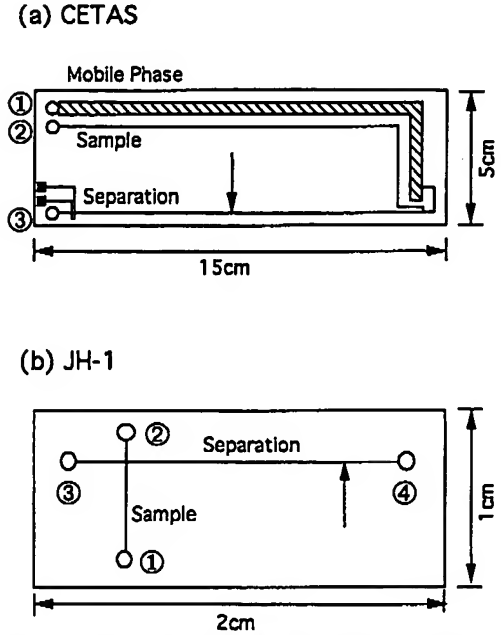

Fig. 1. Layout of two devices fabricated in glass. (a) CETAS device, showing channels and overall dimensions, (b) device JH-1. The contact reservoirs are numbered for reference in the text, and the approximate location of the detector is shown with an error.

The high voltage power supplies and computer control system used has been described elsewhere [9,12]. A 488 nm air-cooled, Ar ion laser (Uniphase/Cyonics) operated at about 4 mW served as a fluorescence excitation source. A 30 cm focal length lens focused the beam into the capillary channel, and a 10:1 microscope objective mounted at 45° to the excitation beam was used to collect emitted light and direct it onto a Hamamatsu R1477 photomultiplier tube (PMT). A 100 μm slit was positioned at the image focal plane of the objective, along with an Omega 505 to 538 nm bandpass filter. The PMT signal was electronically filtered with a 6-pole Butterworth, active filter with a 40 ms (with CETAS) or 5 ms (with

JH-1) time constant and then digitally acquired with a National Instruments NB-MIO-16 board mounted in a Mac II, as described elsewhere [12].

RESULTS AND DISCUSSION

The size and geometric layout of two devices made in glass are shown in Fig. 1, and the channel contact points (reservoirs) referred to in the text by number are indicated. The channels of the CETAS device were etched to a depth of 10 μm and the small channels were 30 μm wide, while the larger channel was 1 mm wide [9]. In the other device, JH-1, all the channels were 10 μm deep and 30 μm wide. CETAS was used to test sample injection and separation efficiencies, while JH-1 was used for rapid separations.

Current–voltage curves in CETAS, with pH 8, 50 mM Tris, 50 mM boric acid buffer, were linear up to at least 10 kV with a voltage applied between reservoirs 1 and 3, indicating no joule-heating effects were observed. This corresponds to 650 V/cm within the separation capillary, two- to three-fold higher than is typically used in CE. For the 6.0 cm separation distance used in these studies this means the maximum potential between the point of injection and detection in CETAS was 3900 V. In device JH-1 linear current voltage curves were obtained for up to 4 kV across the separation channel (potential applied between reservoirs 3 and 4) and 2 kV in the sample channel (reservoirs 1 and 2). This corresponds to up to 2500 V/cm across either channel before Joule heating effects cause non-linearity in the current response due to a temperature rise. For the separation channel in JH-1 a maximum of about 1900 V was dropped across the 0.75 cm between the injection and detection points.

Figure 2 shows the separation of two dyes, fluorescein and (5- or 6-)fluorescein sulfonate in the CETAS structure. By introducing sample into reservoir 2 and applying a voltage between reservoirs 2 and 3 it was possible to inject plugs of sample into channel 3. The length of the plug was readily controlled by the injection voltage and time of application [9]. Fluorescein is readily separated from the more negatively charged sulfonate derivative as shown in the figure, with a combined injection and separation time of 180 s. The fluorescein sulfonate was separated into two components. These may be the two different isomers, or one peak may represent an impurity in the material. Despite this uncertainty, the separation of the two dyes is clearly effected in the planar glass device.

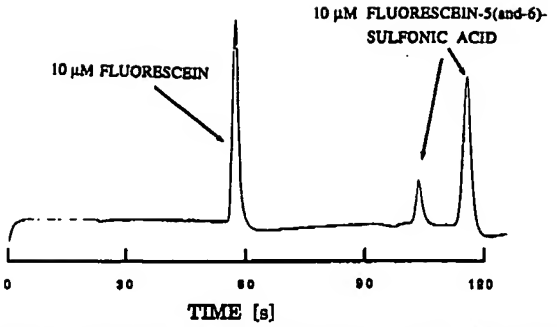

Fig. 2. Electropherogram of fluorescein and isomers of fluorescein sulfonate in pH 8.0 buffer separated in the CETAS device. Sample was injected with 110 V applied for 60 s between reservoirs 2 and 3, followed by a 10 s delay. An 8 kV separation voltage was then applied between reservoirs 1 and 3.

Quantitative sample injection was achieved over a large range of sample volumes in the CETAS device, as shown in Fig. 3. The length of the zone was calculated from the measured electrophoretic mobility of each component, the ap-

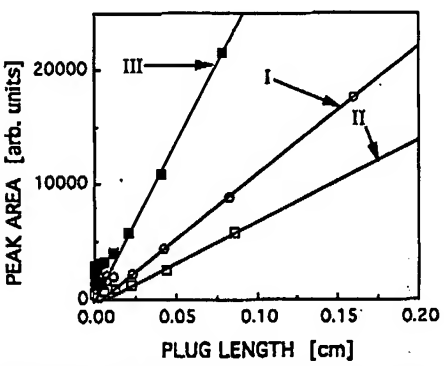

Fig. 3. Peak area (arbitrary units) as a function of plug length for fluorescein (I) and the first (II) and second (III) peaks arising from 5- (and 6)-fluorescein sulfonate in the CETAS device. Injection voltage and time were varied to change the plug length, but the total injection and delay time was 70 s. The separation voltage was constant at 4 kV.

plied voltage and the period of injection. The figure shows there is a linear relationship for sample zone lengths longer than about 200 μm (ca. 60 pl or greater injection volume). Below this value the amount injected decreases in an approximately linear fashion, but with a much lower slope. This effect has not been examined in detail yet, but it must arise at least in part from sample diffusion effects during the period of injection and any delay period between separations. It should be noted that the quantitative relationship *between* the three components is skewed by the fact they move at different velocities, so that the integration times for peaks are different at different migration times. This effect has been discussed [13] and is well understood.

The efficiency of separation in CETAS was evaluated for all three peaks. The height equivalent to a theoretical plate, H, is given by [9]

$$H = \frac{2Dt_m}{d} + \frac{W_{inj}^2}{12d} + \frac{W_{det}^2}{12d} + \frac{2Dt_{inj}}{d} \quad (1)$$

where D is the analyte diffusion coefficient, t_m is the migration time of the peak, t_{inj} is the period of sample injection and an additional delay of 10 s before separation begins, and d is the distance from injection point to detection point. W_{det} and W_{inj} are the detector cell and injected plug lengths. The equation is derived assuming longitudinal diffusion is the only contribution to band broadening within the capillary, and the injector and detector volumes are rectangular [13,14]. Diffusion of the sample plug during injection or delay periods will also contribute to dispersion and so this is also considered.

A plot of observed H versus t_m is shown in Fig. 4 for the components separated in the CETAS device. The value of t_m is inversely dependent on the applied voltage, which covers a range of 3.5 to 10 kV for the data shown (230 to 650 V/cm). The two components of fluorescein sulfonate show a near linear dependence of H on t_m, with a common intercept within experimental error, except at the highest applied voltages (shortest t_m). The intercept is not consistent with the indicated contributions to band broadening. For the estimated values of W_{det} (10 μm), W_{inj}

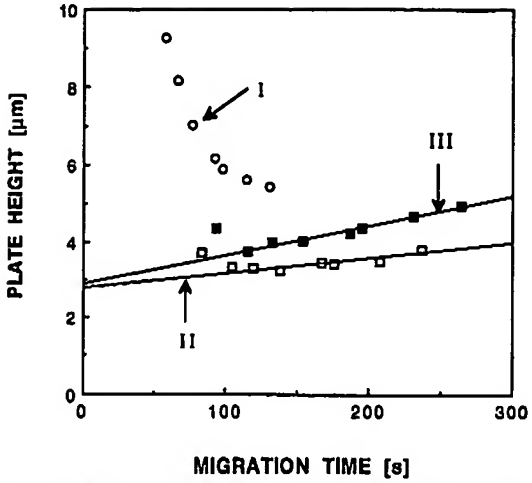

Fig. 4. Measured plate height plotted versus the peak migration time in the CETAS device for fluorescein (I) and the first (II) and second (III) peaks arising from 5- (and 6)-fluorescein sulfonate. The separation voltage varied from 3.5 to 10 kV, with constant injection conditions (110 V, 60 s) and a 10 s delay, in pH 8.0 buffer.

(ca. 200 μm), D of ca. 2×10^{-6} cm^2/s (obtained from the slope of the plots in Fig. 4), and t_{inj} of 70 s the calculated intercept is 0.6 μm. This compares poorly with the actual intercept of 2.8 μm shown in Fig. 4. At the shortest times the bands became notably asymmetric and exhibited tailing, which accounted for the obvious increase in H. The peaks for fluorescein tailed even more at the higher voltages, resulting in very significant H values and the unexpected trend with migration time shown in Fig. 4. These deviations from theory may arise from adsorption of the components on the capillary walls [13], although further study is required to evaluate the effect.

In a previous study [9] of the separation of fluorescein and calcein in a CETAS device we reported nearly ideal separation efficiency, and a maximum number of plates of about 19000 for fluorescein and about 35000 for calcein. In that study the voltage range utilized was 1 to 5 kV, the pH was 8.5 (Tris, boric acid buffer) and the detector provided the most significant contribution to band broadening. In the present study the voltage range was higher, and the detector made no significant contribution. It is clear from this work that additional band broadening effects

would begin to show up only near the maximum potential range used in the previous study.

The data for fluorescein sulfonate shows that relatively high efficiencies can be obtained. For the latest eluting peak H was approximately 4 μm, corresponding to about 15000 theoretical plates across 6 cm. This efficiency was achieved for electric fields ranging from 220 to 400 V/cm. This indicates that a metre long column in a planar glass substrate could give about 250000 plates with 22 kV or more applied. This is comparable to conventional CE in fused silica capillaries, although values close to 10^6 in an open fused silica capillary have been reported.

Figure 5 shows a very rapid separation of the same dye mixture in device JH-1. Sample was introduced into the device in reservoir 1 and driven along the channel to reservoir 2 with 500 V applied. The plug of sample at the intersection of the channels (a geometric volume of 9 pl) was then separated by application of a voltage between reservoirs 3 and 4. The migration time of the peaks decreased linearly with the inverse of the applied voltage, as expected [9,13,14]. Fig. 5 shows that the two dyes were separated within 3 s at an applied potential of 3000 V (1875 V/cm). The two components of the second peak were not resolved, accounting for the peak's broadness. The sample injection time was 1 s, leading to a total analysis cycle time of under 4 s. We found that the magnitude of the injection voltage between reservoirs 1 and 2 affected the peak area upon separation, as did the period of time the potential was applied. Additional study of this is underway, but the data clearly indicate the injected plug is much larger than the geometric volume. Furthermore, if the geometric size of the intersection is assumed to define W_{inj} as 30 μm, while the terms in Eqn. 1 are used to calculate the intercept of H versus t_m, the result is substantially smaller than the 7 μm intercept evident in Fig. 6. Using D for fluorescein of 3.3×10^{-6} cm^2/s [9], $t_{inj} = 1$ s, $W_{det} = 14$ μm and d = 0.75 cm gives a calculated intercept of only 0.1 μm. This is a much greater discrepancy than observed with CETAS. The most probable reason is that diffusion and mixing effects at the intersection mean that the geometric volume is a significant underestimate of the actual volume.

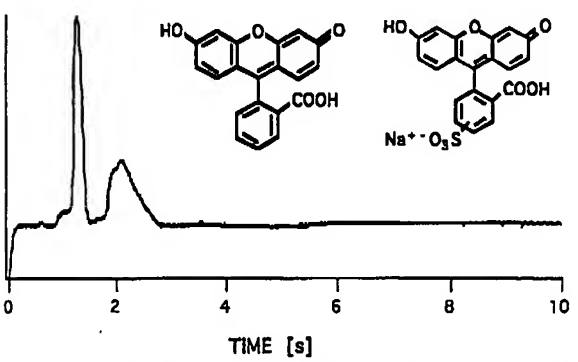

Fig. 5. Rapid separation of 50 μM fluorescein from 50 μM fluorescein sulfonate in device JH-1. Injection was accomplished by applying 500 V for 1 s between reservoirs 1 and 2, and then switching to apply 3000 V between reservoirs 3 and 4 in the pH 8.0 buffer.

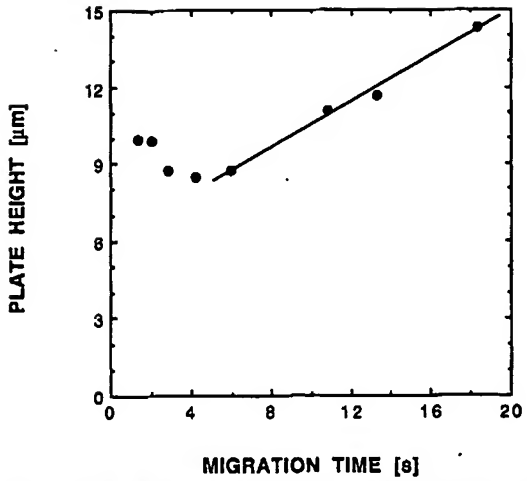

Fig. 6. Measured plate height plotted versus migration time in device JH-1. The separation voltage varied from 200 to 3000 V, with constant injection conditions of 500 V for 1 s between reservoirs 1 and 2.

The number of plates obtained for fluorescein in device JH-1 ranged from 520 at 200 V (125 V/cm) across the separation channel, to 900 plates at 1000 V (625 V/cm), and 780 plates at 3000 V (1875 V/cm). A plot of H versus t_m for fluorescein in this device is shown in Fig. 6. At longer times H depends linearly on t_m as predicted by the equation, but it plateaus and then increases at very short times, just as seen in the CETAS device (Fig. 4). Overall, the efficiency in

device JH-1 is much lower than that observed in CETAS. This is due to the shorter capillary lengths in JH-1, and the consequently greater contribution to band broadening from "off-separation capillary" effects [13–15]. Preliminary work indicates it is possible to reduce these effects by optimizing the injected plug size.

Conclusions

The difference in separation efficiency of the two devices demonstrates the classic trade-off in separation methods between speed and efficiency. The results in the larger CETAS structure show that efficient separations can be achieved in glass substrates. The smaller device shows that very rapid separations can be achieved in these structures. The rapid separations mean the devices could be coupled with flow injection analysis or another separation method such as liquid chromatography to create two dimensional separation [16] or sample handling systems. Similarly, the cycle time is comparable to the response time required in chemical sensor applications, making these systems competitive with chemical sensors.

The devices studied include sample injectors integrated into the capillary column. The ability to control sample flow by applied voltages in the T and cross intersections utilized demonstrates the feasibility of using electroosmotic flow for more complex sample handling within a manifold of capillaries. Micromachining of such manifolds, and electroosmotic pumping of fluid can clearly be used to provide low dead volume systems for handling very small volumes of samples, reagents and carriers in a liquid system in a precise manner.

We thank the Natural Sciences and Engineering Research Council of Canada and Ciba-Geigy for support of the work performed at the University of Alberta. We thank G. MacKinnon of the Alberta Microelectronic Centre (AMC) for valuable assistance in device fabrication, and the AMC for use of their facilities.

REFERENCES

1 N. Graber, H. Lüdi and H. M. Widmer, Sensors Actuators, B1 (1990) 239.
2 M. Gisin and C. Thommen, Anal. Chim. Acta, 190 (1986) 165.
3 M. Garn, P. Cevy, M. Gisin and C. Thommen, Biotechnol. Bioeng., 34 (1989) 423.
4 A. Manz, N. Graber and H. M. Widmer, Sensors Actuators, B1 (1990) 244.
5 A. Manz, J.C. Fettinger, E. Verpoorte, H. Lüdi, H.M. Widmer and D.J. Harrison, Trends Anal. Chem. 10 (1991) 144.
6 J. Ruzicka and E.H. Hansen, Anal. Chim. Acta, 161 (1984) 1.
7 H.M. Widmer, J.F. Erard and G. Grass, Int. J. Environ. Anal. Chem., 18 (1984) 1.
8 A. Giorgetti, N. Periclés, H.M. Widmer, K. Anton and P. Dätwyler, J. Chromatogr. Sci., 27 (1989) 318.
9 D.J. Harrison, A. Manz, H. Lüdi and H.M. Widmer, Anal. Chem., 64 (1992) 1926.
10 A. Manz, D.J. Harrison, E.M.J. Verpoorte, J.C. Fettinger, A. Paulus, H. Lüdi and H.M. Widmer, J. Chromatogr. 593 (1992) 253.
11 Z. Fan and D.J. Harrison, Anal. Chem., submitted for publication.
12 K. Seiler, D.J. Harrison and A. Manz, Anal. Chem., (1993) in press.
13 X. Huang, W.F. Coleman and R.N. Zare, J. Chromatogr., 480 (1989) 95.
14 J.C. Sternberg, Adv. Chromatogr., 2 (1966) 206.
15 J.W. Jorgenson and K.D. Lukacs, Anal. Chem., 53 (1981) 1298.
16 C.A. Monnig and J.W. Jorgenson, Anal. Chem., 63 (1991) 802.